Iranian Journal of Veterinary Science and Technology
Vol. 2, No. 2, 2010, 93-100

IJVST

Investigation of the Antibacterial Effect of Venom of the Iranian Snake *Echis carinatus*

Atena Jami al ahmadi [1], Behrooz Fathi [1]*, Abdoula Jamshidi [2], Hosein Zolfagharian [3] and Abbase Zare Mirakabbadi [3]

[1]*Department of Basic Sciences and* [2]*Department of Food Hygiene and Aquaculture, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
[3] *Razi Vaccine and Serum Research Institute, Tehran, Iran*

Received: August 28, 2010 Accepted: November 8, 2010

Abstract

Although some venoms and their isolated compounds have been shown to have antibacterial properties, most have not been investigated for such activity. *Echis carinatus* is one of the most venomous snakes in the world and has an effective haematotoxic venom that destroys endothelial cells and causes haemorrhagia.

In this study, the antibacterial activity of Iranian snake *Echis carinatus* venom against six different bacteria (*Staphylococcus aureus*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes*, *Bacillus subtilis*, *Salmonella typhimurium* and *Escherichia coli* O157:H7), were investigated. Crude venom (100µg/ml) and different standard antibiotic disks as positive controls were tested by the gel diffusion method. Since the results showed that Echis carinatus venom has a significant antibacterial effect against S. aureus and MRSA, the minimum inhibitory concentrations (MIC) were also determined for these two susceptible bacteria: this was 80µg ml^{-1} against both strains. Also, the results determined that *Echis carinatus* venom dose not have a noticeable effect on other tested bacteria.

Keywords: antibacterial, venom, *Echis carinatus.*

*Corresponding author: Behrooz Fathi
Email: behrooz840@yahoo.com
Telfax: +98 511 876 3655
Mobile: +98 915 976 5651

Introduction

The discovery of penicillin by Alexander Fleming in 1928 changed the world of medicine. From that time, the challenge between bacteria and antibiotics started and still continues (Raghunath, 2008). The majority of bacteria such as *Pseudomonas, Klebsiella, Entrobacter, Acinobacter, Salmonella, Staphylococcus,* Methicillin Resistant *Staphylococcus aureus* (MRSA), *Enterococcus* and penicillin-resistant *Streptococcus pneumoniae* (PRSP) vancomycin-resistant *enterococci* have developed several ways to resist antibiotics. Such bacteria are becoming a serious clinical problem throughout the world (e.g., Ang *et al.*, 2004). Therefore, the discovery of new effective antibacterial agents or developing antibacterials with a new mechanism is continuously necessary.

Natural products are an important source of medicinal compounds. A wide variety of organisms produce such bioactive compounds and some of these natural substances have been shown to be able to kill bacteria (Wenhua *et al.*, 2006; Jensen *et al.*, 2006; Perumal Samy *et al.*, 2006 and Shittu *et al.*, 2007). Snake venoms contain a great variety of biologically active proteins responsible for various pathological effects. Venoms include toxins which are high potency compounds with selective and specific activities. They can be useful and valuable as pharmacological tools in drug research, as potential drug design templates and as therapeutic agents (Harvey *et al.*, 2004 and Koh *et al.*, 2006). In recent years, venoms and venom components from different venomous animals have shown potential antibacterial activity. This includes snake (Stiles *et al.*, 1991 and Perumal Samy *et al.*, 2007) and scorpion venoms (Haeberli *et al.*, 2000; Conde *et al.*, 2000 and Tores-Larios *et al.*, 2002). In addition, venom of the common honey bee (*Apis mellifera*) displays antimicrobial properties (Fennell *et al.*, 1967, 1968). Its major component, mellitin, is more active against gram-positive than gram-negative bacteria. Also, venom of wasps has been reported to have antibacterial properties (Dani *et al.*, 2003 and Perumal Samy *et al.*, 2007), and spider peptide toxins have also been recognized for their antimicrobial properties (Yan and Adams, 1998; Corzo *et al.*, 2002; Budnik *et al.*, 2004; Benli and Yigit, 2008). Recently described potent antimicrobial peptide Latarcin 2a (Ltc2a) from *Lachesana tarabaevi* spider venom showed a broad-spectrum antibacterial activity (Kozlov *et al.*, 2006).

To date, only a few studies have been made on the antimicrobial activities of snake venoms (Perumal Samy *et al.*, 2006). In 1948, Glaser investigated antibacterial activity of *Crotalus* venom (Glaser, 1948) and then in 1968 Aloof Hirsch and his colleagues reported an antibacterial lytic factor from the venom of the cobra *Hemachatus haemachatus* (Aloof-Hirsch, 1968).

Echis carinatus is one of the most venomous viper snakes in the world, found specifically in India, Pakistan, Afghanistan and Iran (Backshall, 2007). It has also shown a potential therapeutic ability. Echistatinas and Ecarin are two medicinal drugs, isolated from *Echis carinatus* venom. Echistatin is one of the most potent disintegrin polypeptide which has platelet aggregation inhibitor activity and used as an anticoagulant while Ecarin is an enzyme used in the ecarin clotting time (ECT) test to monitor anticoagulation during treatment with hirudin. (Garsky *et al.*, 1989; Tans and Rosing, 2001; Guerranti *et al.*, 2002; Nowak G., 2004 and Gawade, 2007).

The aim of the present study was to investigate the antibacterial activity of *Echis carinatus* crude venom against Gram-negative bacteria (*E.coli O157:H7, Salmonella typhimurium*), as well as Gram-positive (*Staphylococcus aureus, Methicillin-Resistant Staphylococcus aureus (MRSA), Bacillus subtillis*, *Listeria monocytogenes)* bacteria.

Materials and Methods

Venom:

Lyophilized crude venom of *Echis carinatus*

was a kind gift of the Razi Vaccine and Serum research Institute, Tehran-Iran.

Micro-organisms

The Gram-positive bacteria including *Staphylococcus aurues* (ATCC: 25923), *Listeria monocytogenes* (ATCC: 7644), *Bacillus subtilis* (ATCC: 6633) and Gram-negative bacteria including *Salmonella typhimurium (ATCC: 14028) and E.coli O157.H7* (ATCC: 35150) were purchased from Mast Laboratories LTD-UK and *Methicillin-Resistant Staphylococcus aureus* (MRSA) bacteria obtained from RCC (Razi Culture Collection).

Standard antibiotics

The standard antibiotics including tetracycline, neomycin and streptomycin were purchased from Liofilchem S.r.l Diagnostic Company (Italy) and used for comparison with the venom.

Methods

Disc-diffusion

Lyophilized crude venom (100 µg) was dissolved in 1ml of 50mM Tris-HCl buffer (pH 7.4) and stored at 4°C for the assay. Antibacterial susceptibility tests were performed by the disc-diffusion method (Bauer *et al.*, 1966). Pure cultures were prepared by subculturing the test strain into 10 ml of Brain Heart Infusion broth (BHI broth) (Merck), following incubation at 37°C for 24 h. The concentration of the resulting culture was determined by preparing serial dilutions and surface plating on SPC agar (Merck). The culture media was diluted and adjusted to 0.5 McFarland standards, containing $1.5x10^8$ cfu ml $^{-1}$, in order to inoculate the same dose of bacteria in repeating the experiment. The absorbance of the cultured media was also determined at 600 nm, using a spectrophotometer apparatus (Jenway 6105, Essex, England).

A sterile cotton swab was used for spreading diluted cultures on Mueller-Hinton (MH) agar plates. Sterile blank paper discs (7mm diameter) were then placed on MH agar surface and 20µl of venom sample were added per disc in five replicates. Antibiogram disks including, Streptomycin (10 µg/disk), tetracycline (30 µg/disk) and neomycin (30 µg/disk) were used as positive controls. The plates were incubated at 37°C for 24h and the zones of inhibition were measured. The experiments were performed at least in seven replicates.

MIC

Minimum inhibitory concentration (MIC) of *Echis carinatus* venom was determined by broth tube dilution technique (Wu and Hancock, 1999). Cultures of bacteria were prepared by inoculating of stock bacteria on MH agar at 37C° for 24h. In the Muller Hinton broth (MHB) 3-4 colonies were grown to a mid logarithmic phase with absorbance of 0.1 at 600 nm (equal to 1.5×10^6 CFU ml^{-1}).

The venom was prepared in the range of 1.25-160 µg ml^{-1} concentrations using Tris-HCl buffer (pH 7.4) (1mol l^{-1}). Twenty micro liter of reconstituted venom was added to 200µl of mid-logarithmic phase of bacterial culture in a 96 well plate.

The bacterial control was made by adding 200 µl inoculated bacterial culture in one well, while negative control was prepared by adding 20 µl of 1mol l^{-1} of Tris-HCl buffer (pH 7.4) to 200 µl uninoculated MH broth.

In order to compare the antibacterial effect of the venom with a standard antibiotic, 20µl of tetracycline solution in Tris-HCl buffer with the same concentrations of the venom (from 160 to1.25 µg ml^{-1}), were added to 200µl of cultured media. The microplates were incubated at 37°C for 24h. The inhibition of bacterial growth was determined by ELISA reader apparatus (ELx 800-Bio-TEK Instrument, INC) measuring the absorbance at 560 nm.

Results

In order to evaluate the antibacterial effects of *Echis carinatus* venom, we used the disc-diffusion method with 20 µl of venom (100

µg/ml) and also different standard antibiotics. In five independent experiments, the venom has shown no antibacterial effects against *E.coli* o157:H7, *Salmonella typhimurium*, *Listeria monocytogenes* and *Bacillus subtilis.* However, standard antibiotics were shown to be effective (Table 1).

In contrast, the venom was effective against *Staphylococcus aureus* and MRSA: maximum inhibitory zones were 11 and 8mm on average, respectively, in at least seven experimental repeats, while the average of inhibition zones of standard antibiotics on both of these two bacteria were 25, 20 and 15 mm for tetracycline, neomycin and streptomycin respectively (Table 1, Figures 1 and 2).

The minimum inhibitory concentration (MIC) determined on the susceptible bacteria, *Staphylococcus aureus* and MRSA, was 80µg ml^{-1} of crude venom for both strains. The assays were performed in five replicates.

Table 1: *In vitro* antibacterial activity of *Echis carinatus* crude venom tested by disc-diffusion and compared to some standard antibiotics. Each number is presented as mean ± SD of inhibition zone in mm, (n =7).

Microorganism Antibiotics / Venom	*L. monocytogenes*	*S. typhimurium*	*B. subtilis*	*E.coli O157.H7*	MRSA	*S. aureuss*
E. carinatus Venom	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.13	8.2 ± 0.5	11.09 ± 2.3
Tetracycline	25 ± 0.0	25 ± 0.0	25 ± 0.0	25 ± 0.0	25 ± 0.0	25 ± 0.0
Neomycin	25 ± 0.0	25 ± 0.0	25 ± 0.0	20 ± 0.0	20 ± 0.0	20 ± 0.0
Streptomycin	15 ± 0.0	15 ± 0.0	15 ± 0.0	15 ± 0.0	15 ± 0.0	15 ± 0.0

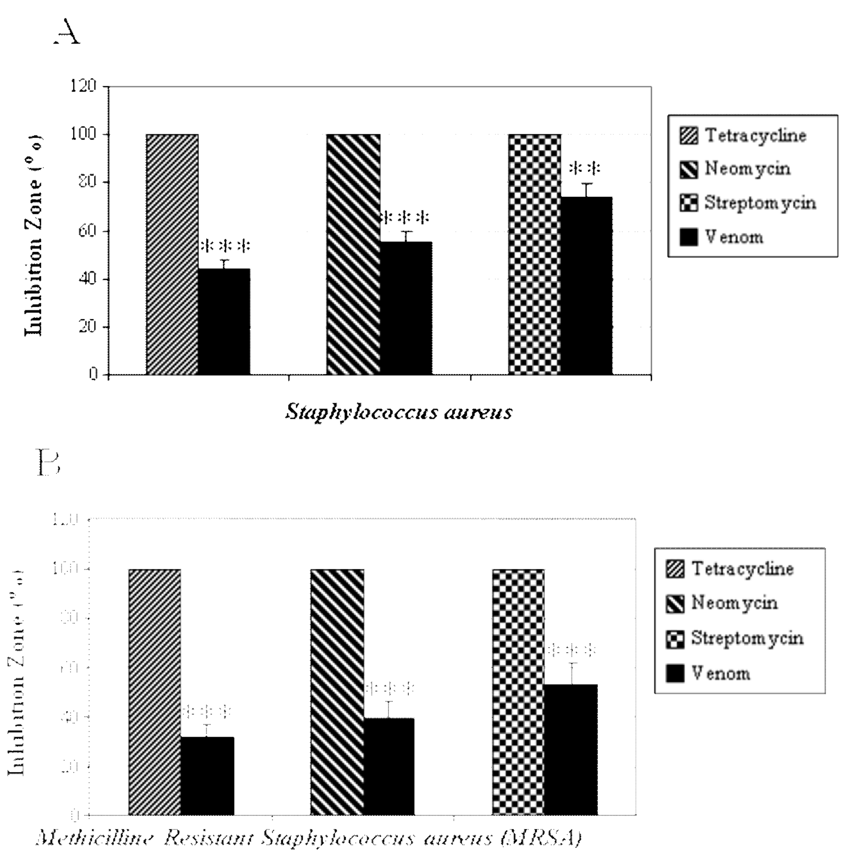


Figure 1: Antibacterial effect of *Echis carinatus* venom against *Staphylococcus aureus* (A) and *Methicillin Resistant Staphylococcus aureus (MRSA)* (B), in compare with three different standard antibiotics, tetracycline, neomycin and streptomycin

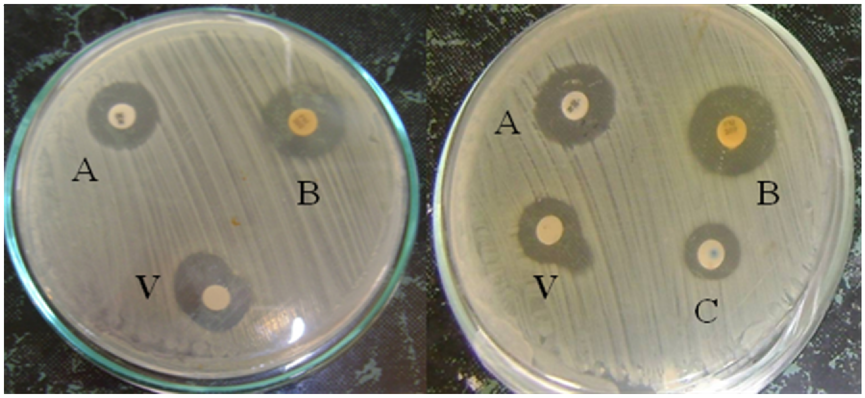


Figure 2: Antibacterial effect of *Echis carinatus* venom (V) against *Staphylococcus aureus* (Left) and *Methicillin Resistant Staphylococcus aureus (MRSA)* (Right), in compare with two and three different standard antibiotics; neomycin (A), tetracycline (B), streptomycin (C).

Discussion

Researchers have reported antibacterial effects of some venoms including *Echis carinatus* venom against some bacteria (Perumal Samy *et al.*, 2006; 2007). Herein it was clear that *Echis carinatus* venom has not a wide spectrum antibacterial effect against the mentioned bacteria, although a significant activity against *S. aureus* and MRSA in comparison with the standard antibiotics, tetracycline, streptomycin and neomycin has been observed. This is in agreement with the results of Gopalakrishnakone (Perumal Samy *et al.*, 2006).

As described in the literature, venom consists of many different substances like proteins and enzymes. Which responsible for its biological activities. Therefore, these compounds may interact with specific molecules of some bacteria while not affecting other strains. Herein we conclude that the venom of *Echis carinatus* lacks effective proteins responsible for its antibacterial activity for some specific strains while it was effective against *S. aureus* and MRSA.

It has been reported that phospholipase A_2 (PLA_2) can have antibacterial effects (Nunez *et al.*, 2004; Barbosa *et al.*, 2005; Perumal Samy *et al.*, 2006 and Xu *et al.*, 2007), while *Echis carinatus* venom contains PLA_2 activity (Kemparaju *et al.*, 1994), and this may be responsible for its antibacterial properties. However, Ferreira and his colleagues reported that, *Lachesis muta* venom had PLA_2 activity, with no antibacterial effect. They concluded that presence of PLA_2 in the venom does not guarantee the antibacterial activity. Therefore, it is possible that *Echis carinatus* venom may have a specific mechanism or unknown molecule that exhibit antibacterial effect on the susceptible bacteria.

To determine whether *Echis carinatus* venom can influence other pathogens, further studies are needed using a wider spectrum of Gram-positive and Gram-negative bacteria and also other concentrations of this venom. Fractionated venom for detection of active components can improve investigation of its antibacterial activity.

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Iranian Journal of Veterinary Science and Technology
Vol. 2, No. 2, 2010, 93-100

IJVST

بررسی اثر ضد باکتریایی زهر مار جعفری ایران

آتنا جامی الاحمدی[1]، بهروز فتحی[1]، عبدا.جمشیدی[2]، حسین ذوالفقاریان [3] و عباس زارع[3]

گروه های [1] علوم پایه و [2] بهداشت مواد غذایی و آبزیان ، دانشکده دامپزشکی، دانشگاه فردوسی مشهد، مشهد، ایران
[3] موسسه تحقیقات واکسن و سرم سازی رازی، کرج، ایران

دریافت مقاله: ۸۹/۶/۶　　　*پذیرش نهایی: ۸۹/۸/۱۷*

چکیده

اگر چه برخی از زهرها و ترکیبات مشتق شده از آنها، نشان داده اند که دارای خواص ضد باکتریایی هستند اما بیشتر آنها برای یافتن چنین فعالیتهایی مورد بررسی قرار نگرفته اند. در دنیای فارماکولوژی، همزمان با کشف و مصرف آنتی‌بیوتیک‌های جدید، باکتری‌ها نیز واجد ویژگی‌هایی می‌شوند که آنتی‌بیوتیک‌ها بر آن‌ها بی‌اثر بوده و مسئله مقاومت باکتریایی مطرح می‌گردد. این امر باعث تحقیق و بررسی بیشتر دانشمندان در منابع طبیعی مختلف برای کشف آنتی‌بیوتیک‌های موثرتر و جدیدتر می‌شود. زهرهای جانوران به دلیل اثرات مختلف مشاهده شده از جمله اثر آنتی‌باکتریال بسیار مورد توجه محققین قرار گرفته‌اند. زهر ترکیب بسیار پیچیده‌ای از انواع پپتیدها و مواد غیر پپتیدی با فعالیت‌های گوناگون می‌باشد. مطالعات بسیار کمی برای بررسی اثر آنتی‌باکتریال و خالص‌سازی زهر مارها صورت گرفته است. در این مطالعه اثرات آنتی‌باکتریال زهر مارجعفری ایران برعلیه شش گونه باکتری از جمله: استافیلوکوکوس اورئوس، استافیلوکوکوس اورئوس مقاوم به متیسیلین، اشریشیا کولای، سالمونلا تایفی موریوم، باسیلوس سوبتیلیس و لیستریا مونوسایتوژنز با استفاده از تست انتشار در دیسک و تست بررسی حداقل غلظت ممانعت کننده از رشد، مورد بررسی قرار گرفت. در این آزمایشات از سه آنتی‌بیوتیک استرپتومایسین، نئومایسین و تتراسایکلین نیز به عنوان کنترل مثبت استفاده گردید. نتایج نشان داد که زهر مار جعفری ایران دارای اثر آنتی‌باکتریال بوده و بر باکتری-های استافیلوکوک اورئوس واستافیلوکوک اورئوس مقاوم به متیسیلین بطور معنی داری موثر می‌باشد.

واژگان کلیدی: ضد باکتری، زهر، مار جعفری